Trans R Soc Trop Med Hyg 2020; **114**: 858–865
doi:10.1093/trstmh/traa055 Advance Access publication 7 August 2020

REVIEW ARTICLE

Polymorphism in the IL-1β promoter is associated with IgG antibody response to circumsporozoite protein repeats of *Plasmodium vivax*

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Received 19 February 2020; revised 18 May 2020; editorial decision 3 June 2020; accepted 2 July 2020

Background: It is well established that infection by *Plasmodium vivax* is a result of host-parasite interactions. In the present study, association with the IL1/IL2 cytokine profiles, anticircumsporozoite protein antibody levels and parasitic loads was evaluated in individuals naturally infected with *P. vivax* in an endemic area of the Brazilian Amazon.

Methods: Molecular diagnosis of *P. vivax* and variants was performed using the PCR-RFLP method and *IL1B -511C>T*, *IL2 -330T>G* and *IL2+114T>G* polymorphisms were identified using PCR-RFLP and allele-specific PCR. IL-1β and IL-2 cytokine levels were detected by flow cytometry and circumsporozoite protein (CSP) antibodies were measured by ELISA.

Results: Three variants of *P. vivax* CSP were identified and VK247 was found to be the most frequent. However, the prevalence and magnitude of IgG antibodies were higher for the VK210 variant. Furthermore, the antibody response to the CSP variants was not associated with the presence of the variant in the infection. Significant differences were observed between the single nucleotide polymorphism (SNP) -511T>C in the *IL1B* gene and levels of antibodies to the VK247 and *P. vivax*-like variants, but there were no associations between SNPs in *IL1* and *IL2* genes and their plasma products.

Conclusions: Individuals with the rs16944 CC genotype in the *IL1β* gene have higher antibody levels to the CSP of *P. vivax* of VK247 and *P. vivax*-like variants.

Keywords: antibodies, circumsporozoite protein, IL-1, IL-2, *Plasmodium vivax*, polymorphisms

Introduction

Malaria caused by *Plasmodium vivax* accounts for approximately 50% of malaria cases that occur outside Africa. In Brazil, the majority of detected cases are caused by this protozoan species.[1] Infections caused by *P. vivax* have a variety of symptoms, ranging from asymptomatic infections to serious complications, such as severe anemia, acute respiratory failure and eventual death.[2] It is well established that infection results from parasite-host interactions and the nature of the generated immune response.

In endemic areas of malaria transmission, individuals with repeated exposure to the parasite tend to develop clinical immunity to both *Plasmodium falciparum*[3] and *P. vivax*[4] malaria. This is a key observation for a conceptual framework in malaria vaccine development. However, despite decades of research, a highly effective vaccine still remains elusive. To date, only one vaccine formulation against *P. falciparum* has been licensed, RTS, S, which showed limited protective efficacy in young children (approximately 36%).[5] This vaccine is based on circumsporozoite protein (CSP), the major surface antigen present in sporozoites, that is

critical in liver-stage development during the pre-erythrocytic life-cycle.[6] CSP has a central immuno-dominant region of tandem repeats flanked by two highly conserved regions that encode the amino terminal and carboxy terminal regions. For *P. vivax,* the most advanced vaccine candidate (phase II clinical trial) also targets CSP.[7] However, unlike *P. falciparum*, the CSP of *P. vivax* (*Pv*CSP) exhibits diversity in the central repetitive domain, which defines the variants known as VK210, VK247 and *P. vivax*-like.[8]

Cross-sectional studies evaluated the naturally acquired antibodies to the *Pv*CSP in individuals from several malaria-endemic areas around the world and the seroprevalence ranged from 25% to 85%, with VK210 being the most predominant variant. [9-11] The reasons for the great inter-individual variation in immune response to malaria, whether naturally acquired or vaccine-induced, are not yet fully understood, but is it probable that the outcome of immune response is a multifactorial process, requiring an interplay between parasite-host and environmental factors. In addition to parasite variations, growing evidence shows that the host genetic background can contribute to marked differences between individuals in the course of infection, thus generating conflicting results in host-parasite interaction studies. It is therefore valuable to identify which genes could be important in the host response to malaria infection, as this could provide insights into the molecular mechanisms involved in the pathogenesis and immunity to malaria. Likewise, it will be possible to pinpoint the factors involved in the immune response regulation, including the polymorphisms in molecule-encoding genes, which are important humoral and cellular immune response mediators.[12–17]

In the present study, we evaluated the potential influence of polymorphisms in the *IL1β* and *IL2* genes on the immune response against *Pv*CSP in naturally exposed individuals. IL-1 has a broad spectrum of biological responses on cells of the immune system,[18] either by regulating T cell proliferation and also by acting as a cofactor in the activation of B cells. IL-1β is involved in antibody production through induction of CD40L and OX-40. Polymorphic variability in the IL-1β gene has already been associated with variations in mumps antibody levels[19] and with autoimmune diseases such as rheumatoid arthritis,[20] Graves' disease[21] and systemic lupus erythematosus.[22] High levels of IL-1β are found in individuals with cerebral malaria and promoter polymorphisms in the *IL1B* gene confer protection against *P. falciparum* severe malaria.[23] On the other hand, in *P. vivax* infections, these variations have been associated with parasitemia and anaemia.[24,25]

IL-2 is mainly produced by activated T cells and it is important for their differentiation into effector and memory T cells. It also acts as a negative regulator of IFN-γ and TNF-α secretion.[18,19] In malaria, IL-2 is produced in response to parasite antigens and regulates the Natural Killer (NK) cell-mediated lysis against schizonts.[12] Furthermore, IL-2 may enhance the antibody production by B cells. There was a strong association between the titers of CSP antibodies and the frequency of IL-2-producing $CD4^+$ cells in RTS, S-immunized individuals, suggesting that this cytokine plays a major role in the humoral immune response.[13] Thus, the objective of this study was to investigate parasite and host polymorphisms that may affect the immune response to *P. vivax*. To this end, the present study aimed to characterize the molecular variants of *Pv*CSP and host genetic polymorphism in *IL1β* and *IL2* genes, and their potential association with naturally acquired antibody responses to *Pv*CSP.

Materials and methods

Study area and population

This study included 131 patients from a previous study conducted in the Goianésia do Pará municipality of Pará, Brazil.[14] This region was among the five municipalities that accounted for 50% of malaria cases in Pará in 2012, where the blood samples were collected. Patients were included in the study after confirmed infection with *P. vivax*, which was diagnosed by thick blood smear. Additionally, confirmatory molecular diagnostic PCR was performed[15] on all the samples included in our study.

Laboratory tests

For the analysis of polymorphisms in the *IL1B -511C>T* (rs16944) gene, the DNA samples were amplified using the PCR-RFLP method according to Cantagrel et al.[16] The size of the resulting amplified fragment was 305 bp, which was digested with restriction endonuclease Eco 881. The presence of the T allele was identified by one 305 bp fragment and the C allele by the presence of 115 and 190 bp fragments. Determination of the single nucleotide polymorphism (SNP) +114T>G (rs2069763) in the *IL2* gene was performed using the PCR-RFLP method according to Song et al.[17] The size of the resulting amplified fragment was 262 bp. After digestion with the *MwoI* enzyme, the G allele was identified by formation of 111 and 151 bp fragments, while there was no T allele cleavage. The determination of the SNP -330T>G (rs2069762) in the promoter of *IL2* gene was performed using the allele-specific PCR with confronting two-pair primers (PCR-CTPP) according to the protocol described by Togawa et al.[26] The resulting G allele fragment was 215 bp, while the T allele fragment was 152 bp.

The *P. vivax* CSP variants were analyzed using the PCR-RFLP method. Amplification was performed as described by Cassiano et al.[27] with modifications. A reaction mix was prepared with a final volume of 25 μL containing *P. vivax* DNA, 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.6 mM MgCl, 0.2 mM each dNTP, 0.2 μM of each primer (5′ AGGCAGAGGACTTGGTGAGA 3′ and 5′ CCACAGGTTACACTGCATGG 3′) and 1 U of Platinum Taq (Invitrogen, EUA). The reaction was carried out in a thermocycler (DNA Mastercycler, Eppendorf, Germany) as follows: an initial cycle at 94°C for 15 min, followed by 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. As a positive control, we used three plasmids containing a gene insertion of the CSP repetitive portion from the variants amplified from *P. vivax* VK210, VK247 and *P. vivax*-like (Bluescript Stratagene, La Jolla, CA, USA). Sterile water was used for the negative control. The digestion reaction was performed in a final volume of 20 μL containing 10 U of *AluI* (Invitrogen), 2 μL of enzyme reaction buffer, 10 μL of PCR product and 7 μL of DNase-free sterile water. The reactions were performed overnight in a water bath at 37°C.

A BD cytometric bead array kit was used in this study to quantify the IL-1β and IL-2 cytokines according to the protocol described by Walther et al.[28] Briefly, sera and cytokine patterns from the kit were incubated with capture beads coated with antibodies specific to the respective cytokines and with detection antibody labeled with phycoerythrin. After incubation, a wash solution was added and centrifuged. The supernatant was discarded and the samples were resuspended for acquisition on a FACSCanto type II flow cytometer (Becton Dickinson, San José, CA, USA). A standard curve was generated for each cytokine and analyzed using FACSDiva software (Becton Dickinson). The results were analyzed using FCAP Array 3.0 software (Becton Dickinson). Raw fluorescence intensity mean values were quantified for each cytokine. Values were expressed in pg/ml for each cytokine in comparison with the standard curve. The measurements were performed in duplicate and the arithmetic mean of the two values was used for analysis.

Anti-CSP antibodies were detected using synthetic peptides corresponding to the immuno-dominant epitope of the repetitive regions of *Pv*CSP, as described by Cochrane et al.[29] Briefly, 96-well plates (Maxsorp) were incubated overnight at 4°C with antigens at concentrations of 1 μg/mL for the VK210 variant and 5 μg/mL for the VK247 and *P. vivax*-like variants. After the incubation period, the antigens were blocked with Bovine Serum Albumin (Sigma-Aldrich, USA). The sera were diluted at a ratio of 1/100 in PBS-T-2.5% BSA and applied to the 96-well plate (Corning, New York, USA) in duplicate. After washing, 100 μL of anti-human IgG conjugate labeled with peroxidase (Sigma, St. Louis, MO, USA) was added to each well. The plates were incubated at 37°C for 1 h. After a second washing, 100 μL of substrate (ortho-phenylenediamine and hydrogen peroxide [Merck, Darmstadt, Germany]) was added to each well and again incubated at 37°C for 1 h. The reaction was stopped with 100 μL of 1% H_2SO_4 solution.

Sera from 21 control (non-malarial) samples from Rio de Janeiro were used as a negative control.[14] The cut-off value was determined to be the mean optical density (OD) + three standard deviations of the control samples (cut-off values: VK210=0.073; VK247=0.060; *P. vivax*-like=0.070). To homogenize the OD data obtained in the different experiments, the reactivity index (RI) was calculated for each sample by dividing the mean OD of the sample tested by the OD cut-off value of each experiment. Samples with RI>1.0 were considered positive.

Statistical analysis

Genotypic and allelic frequencies were estimated by direct counting. Hardy–Weinberg equilibrium (HWE) was evaluated using Bioestat version 5.3. Statistical analyses were performed using R version 3.0.0. The charts were created using GraphPad Prism version 5.01. For univariate analysis, differences in proportions were measured using the χ^2 test and differences in medians were tested using Kruskal–Wallis and Mann–Whitney U tests. Correlations were measured using the Spearman coefficient. Association analyses involving SNPs were performed using the SNPassoc R package.[29] The SNPs were tested following different genetic models (codominant, dominant or recessive) on the basis of generalized linear models using covariates such as age, gender and malaria history. $p<0.05$ was considered significant.

Results

The mean age of the 131 individuals included in our study was 29.8 (SD±14.1) years. Of the 122 individuals who remembered having already contracted malaria in the past, only eight reported never having had a previous episode of malaria. The mean parasitemia was 769.3 parasites/mm^3 (95% CI 494.3 to 1197.4).

The *P. vivax* CSP gene was successfully amplified and single infections containing only the VK247 variant were the most common (45.8%), followed by simple infections with the VK210 varant (36.6%) and mixed infections containing the VK210 and VK247 variants (13.7%). The *P. vivax*-like variant was detected as a single infection in only one sample (0.8%) while mixed infections with VK210 and VK247 were detected in four samples (3%). There was no significant difference in parasitemia between the VK210 variant (geometric mean=460.2 parasites/mm^3, 95% CI 224.3 to 903.6) and VK247 variant (geometric mean=965.9 parasites/mm^3, 95% CI 562.9 to 1657.6) (p=0.20, Mann–Whitney test).

Of the individuals infected with *P. vivax*, 28.2% had antibodies specific to at least one CSP variant. The frequency of responders for the VK210, VK247 and *P. vivax*-like variants was 22.6%, 8.3% and 12.0%, respectively (Figure 1A). The prevalence and RI of CSP antibodies were higher for the VK210 variant than for VK247 (p=0.001). Regarding the magnitude of the IgG response, higher RIs were observed for the VK210 variant (median=0.60; IQ: 0:43–0.87) than for the VK247 (0.39; IQ: 0.30–0.52) and *P. vivax*-like (0.43; IQ: 0.36–0.62) ($p<0.0001$) variants (Figure 1B). There was a significant correlation between IgG antibody levels for all three variants (Figures 1C-E).

In addition, we tested whether demographic variables (age, previous history of malaria and residence time in the study area) could affect the antibody responses to *Pv*CSP and no significant association was observed. There was no significant difference in antibody responses between individuals infected with the three variants. The responders for the VK210 CS peptide were similar among individuals infected with the VK210 variant compared with individuals who were not infected with VK210 (24.3% vs 19.7%, p=0.67). Likewise, the higher frequency of individuals infected with the VK247 variant did not increase the responders to the VK247 CS peptide (8.5% vs 8.2%, p=1.0). Moreover, there was no significant difference in the CSP antibody RI according to the corresponding variant present in the infection (VK210 or VK247). Since only one sample contained a single *P. vivax*-like variant infection, further analysis was not performed.

Cytokine profile was evaluated in 77 of the 131 malaria individuals studied and 21 controls. Figure 2 shows the IL1α, IL-1β and IL-2 cytokine levels. Comparisons of the IL1α levels were not statistically significant (Figure 2A). The IL1β levels were higher in malaria patients than in endemic controls (Figure 2B, $p<0.001$). Of the malaria patients, 24 samples met or exceeded the detection limit for IL-1β (median 10.73 pg/mL; IQR: 4.41–28.32), while only seven samples exceeded the detection limit of IL-2 levels (median 13.35 pg/mL, IQR: 12.1–17.1) and the IL2 levels were significantly higher in endemic controls than in malaria patients (Figure 2C, p=0.004). A positive correlation was observed between IL1α/IL1β levels (Spearman's rho=0.236, p=0.03). There was no significant correlation between

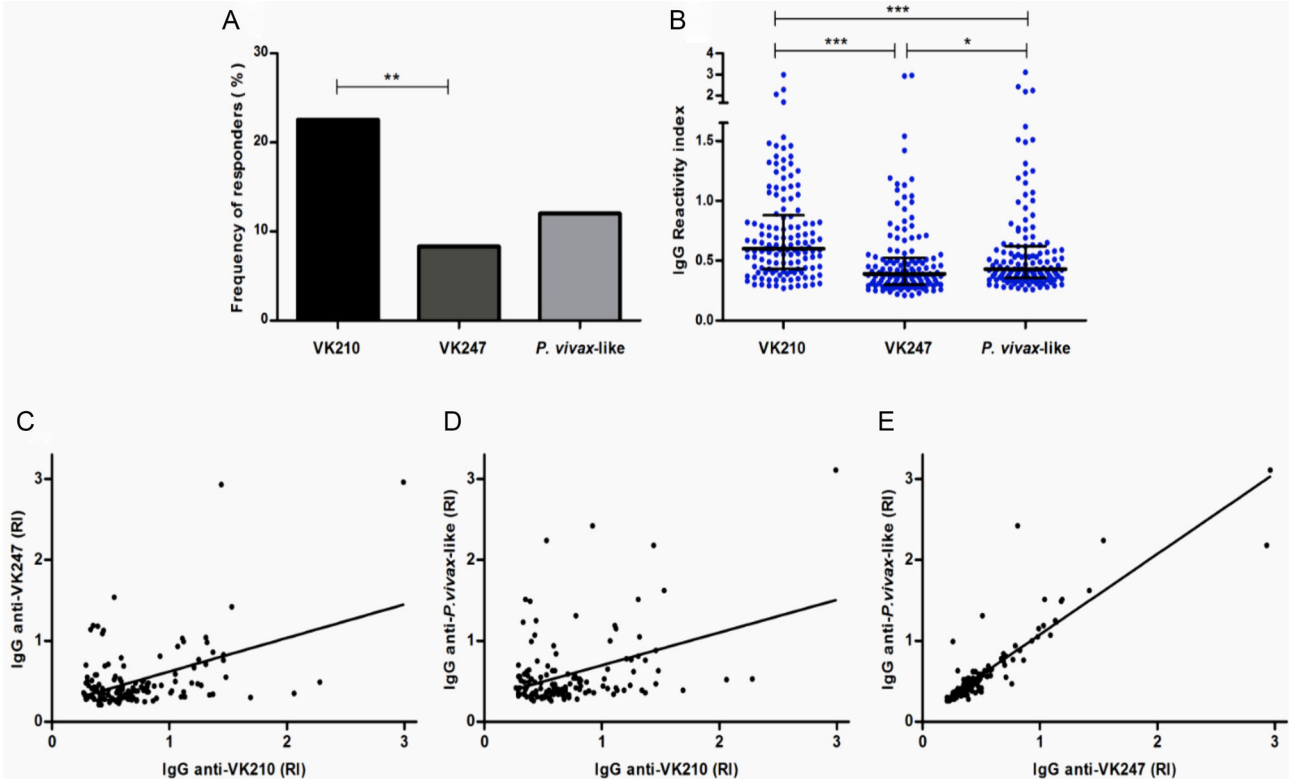

Figure 1. Naturally acquired IgG antibodies against *Pv*CSP variants. (A) Frequency of IgG responders against the three variants of *Pv*CSP. (B) Differences in IgG (RI) antibody levels for the three *Pv*CSP variants (Kruskall–Wallis test, p<0.001). (C) Significant Spearman correlation between levels of specific antibodies against variant VK210 and VK247 (r=0.236, p=0.006); (D) VK210 and *P. vivax*-like (r=0.221, p=0.01) and (E) VK247 and *P. vivax*-like (r=0.874, p<0.0001). *p<0.05; **p<0.01; ***p<0.001.

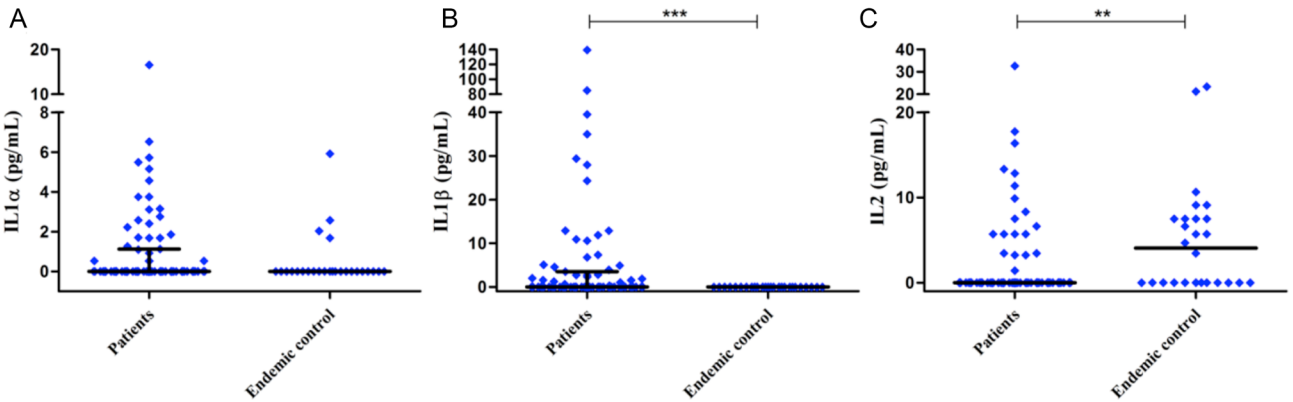

Figure 2. Cytokine profile in the serum of patients infected with *P. vivax* vs endemic controls. The analysis included IL1α (A), IL1β (B) and IL2 (C). Cytokines were measured by flow cytometry. The bars represent the median of the group and interquartile range. p-values were determined by non-parametric Mann–Whitney U tests (**p<0.01, ***p<0.001).

IL1α/IL-2 (Spearman's rho=0.149, p=0.19) and IL-1β/IL-2 levels (Spearman's rho=0.128, p=0.26). There was also no significant correlation between the levels of interleukins with parasitemia and anti-CSP variant antibody levels. There was no significant difference in the levels of IL1α (p=0.48), IL-1β (p=0.89) and IL-2 (p=0.07) between infections containing the VK210 and VK247 variants.

The 77 malaria patients with cytokine profiles were genotyped to verify SNPs in the *IL1B* (rs16944) and *IL2* (rs2069762 and rs2069763) genes. Regardless of the tested genetic model (codominant, recessive or dominant), no significant associations were observed between SNP rs16944 in the *IL1B* gene and IL-1β levels or between SNPs rs2069762 and rs2069763 in the *IL2* gene and IL-2 levels (Figure 3).

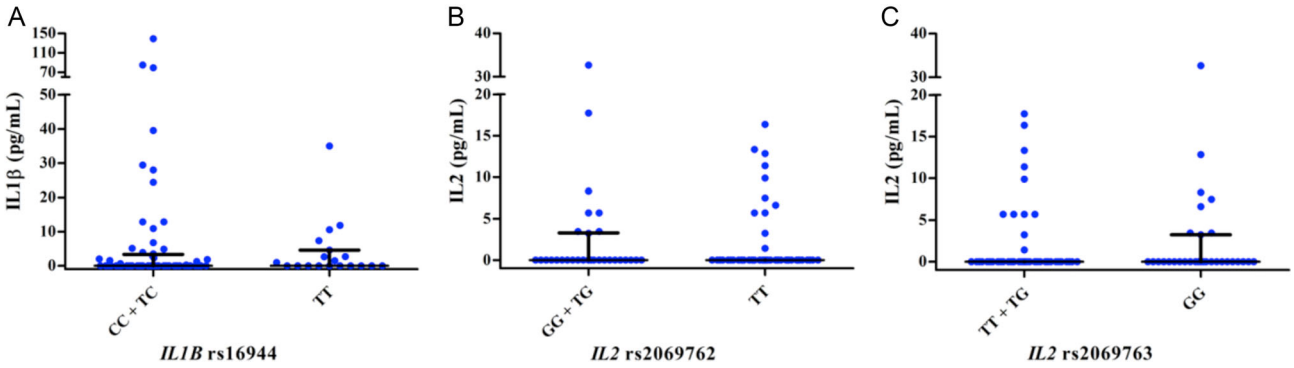

Figure 3. Association between SNPs (A) *IL1B* rs16944, (B) *IL2* rs2069762 and (C) *IL2* rs2069763 with their gene products. The bars represent the median and IQR. There was no significant difference using different genetic models.

Table 1. Frequency of polymorphisms and association with antibody levels against variants of CSP

			VK210		VK247		*P. vivax*-like	
Gene	SNP	n (%)	RI	p[a]	RI	p[a]	RI	p[a]
IL1B	rs16944			0.14		0.005		0.005
	C/C	12 (15.6)	0.66±0.71		0.77±0.76		0.95±0.86	
	C/T	49 (63.6)	0.59±0.29		0.44±0.21		0.53±0.36	
	T/T	16 (20.8)	0.94±0.53		0.56±0.29		0.64±0.34	
IL2	rs2069762			0.54		0.25		0.07
	G/G	3 (3.9)	0.62±0.36		0.37±0.01		0.44±0.03	
	G/T	27 (35.1)	0.81±0.64		0.59±0.53		0.74±0.68	
	T/T	47 (61.0)	0.60±0.30		0.48±0.27		0.55±0.34	
IL2	rs2069763			0.31		0.93		0.99
	G/G	32 (42.1)	0.63±0.52		0.51±0.47		0.61±0.58	
	G/T	40 (52.6)	0.69±0.40		0.52±0.31		0.60±0.41	
	T/T	4 (5.3)	0.84±0.52		0.58±0.33		0.83±0.44	

[a]p-values based on fitting logistic regression models adjusting for gender, age and previous malaria infection. RI: reactivity index.

The SNPs were evaluated with respect to anti-CSP antibody levels. The genotypic frequencies of *IL1B* (rs16944) and *IL2* (rs2069762 and rs2069763) are presented in Table 1. Deviation of HWE was observed only for the SNP rs16944 (p=0.02). Significant differences were observed in the SNP rs16944 in the *IL1B* gene with levels of antibodies to the VK247 and *P. vivax*-like variants. Individuals with the *CC* genotype had higher levels of anti-VK247 antibodies than individuals with *CT* or *TT* (0.77 vs 0.44 and 0.56, respectively, p<0.005). Furthermore, it was observed that individuals with the *CC* genotype also had more anti-*P. vivax*-like antibodies (0.95 vs 0.53 and 0.64, p<0.005).

Discussion

Using serological and/or molecular approaches, researchers have evaluated the occurrence of *P. vivax* CSP variants (VK210, VK247 and *P. vivax*-like) in endemic areas of the Amazon. Both VK210 and VK247 variants are widely distributed.[8,30–32] However, unlike previous reports from different areas of the Brazilian Amazon, the VK247 variant was found in higher frequencies than VK210 in Goianésia do Pará. This finding is particularly important both clinically and epidemiologically because infections with the VK247 variant have a higher parasitic burden and prevalence of inflammatory cytokines, which could result in major complications.[33] The determining factors contributing to differences in the distribution of *P. vivax* CSP variants in endemic areas are unclear but may be related to the epidemiological transmission conditions. The population genetic structure of *P. vivax* is probably influenced by local vector species susceptibility to the parasite variant, which may facilitate the introduction or exclusion of new strains and genotypes with favorable transmission conditions.

The CSP's repetitive central region, which characterizes the *P. vivax* variants, contains information for encoding B cell epitopes. The human host thus generates specific antibodies to this region that are able to block invasion of sporozoites into the hepatocytes[34] correlated with protection.[35] The higher frequencies of VK247 in current infections compared with higher antibody responses to VK210 may suggest that VK210 protein is more immunogenic than VK247, as shown in other studies,[36,37] and

also that the VK210 variant could have been more prevalent in this area in the recent past, as the majority of the patients in our study had a previous history of malaria infections. In fact, molecular methods can detect the genotypes of the current infection while serological techniques detect antibodies, which can be detected for several months and even years after the end of the infection. In addition, it is not known if the hypnozoites/relapses could influence the antibody response to sporozoites since the presence of a blood-stage infection, as in the case of *P. vivax*, may not always indicate a new infection.

Another important result was the detection of anti-*P. vivax*-like variant antibodies. Although this variant was recognized in the early 1990s in Papua New Guinea,[38] several worldwide studies have failed to detect it.[31,39,40] In our study, the detection of so few samples of *P. vivax*-like, as well as antibodies specific to this variant, indicate that *P. vivax*-like circulation occurs at low frequencies in the Pará state. Similar results have been observed in different Amazonian regions in Brazil[41–43] and French Guiana.[44] Brazil is one of the few countries in South America that registers the circulation of this variant. The present results suggest that genetic and immunological factors are not favoring its adaptation in the studied area of the Brazilian Amazon.

Overall, the seropositivity of individuals with *P. vivax* anti-CSP repeat antibodies was low (28.2%, considering the three variants). This is in agreement with data observed in other malaria-endemic areas of the Brazilian Amazon.[42,44,45] In fact, the frequency of *P. vivax* anti-CSP antibodies depends on different factors, including exposure to the parasite, age and the human host genetic background.[42,46,47] Lack of response to all CSP repeats, even in individuals with current infection, is widely reported and shows the association of specific HLA alleles and immune response to malaria antigens.[41] For this reason, the potential association of *IL1B* and *IL2* gene polymorphisms with IgG antibody responses to CSP variants was investigated. Studies in human and murine models show that cytokines play a key role in immune modulation in malaria, participating in parasitemia control and physiopathogenesis of the disease, depending on their cellular and circulating levels.[48]

Here, two polymorphisms in the *IL2* gene (+114T>G and -330T>G) were analyzed. IL-2 may inhibit or enhance antibody production by B cells.[49] However, the allelic variants of *IL2* +114 and -330 showed no association with anti-*Pv*CSP antibody response. Associations of the -330T>G polymorphism with TB,[50] colorectal cancer[51] and hepatitis B/C virus[52] were recently demonstrated, but not for cutaneous leishmaniasis[53] or acute lymphoblastic leukemia.[54] *In vitro* assays demonstrated that this SNP has a role in the production of IL-2,[55] but its association with plasma IL-2 levels is controversial.[51,56] The SNP +114T>G is located in the first exon of this gene and is associated with inflammation-based cancers such as hepatocellular carcinoma[57] and gastric cancer.[56] In malaria, only the SNP -330T>G has been investigated previously[24] and no association with *P. vivax* malaria was found. In this study, the minor allele frequency of allele *G* in *P. vivax*-infected individuals was about 30%, similar to the findings in Goianésia do Pará.

In the present study, we found that individuals with the *CC* homozygous genotype for the SNP *IL1-B* -511C>T (rs16944) showed a higher IgG antibody response to the VK247 and *P. vivax*-like variants. This SNP has recently been studied in various diseases and the T allele has been associated with an increased risk of septic shock,[58] gastric cancer[59] and cervical cancer.[60] In malaria, the allelic frequencies of this SNP did not differ between symptomatic and asymptomatic individuals in the Fulani ethnic group[61] but it has been linked to severe anemia in patients with falciparum malaria in Kenya.[62] Interestingly, in our study, this SNP did not affect IL-1β levels. In fact, previous studies have failed to associate -511C>T (rs16944) with relevant changes in gene transcription.[63,64] This SNP is located upstream of the transcription start site in the gene's promoter region; a high linkage disequilibrium between SNPs located in this region has been demonstrated and changes in gene expression appear to be more affected by haplotypes than by specific SNPs.[63] Therefore, SNP rs16944 is probably not responsible for these changes but may be in linkage disequilibrium with the true causal variant or participates in the context of certain haplotypes. Thus, one of the limitations of this study was the evaluation of a single SNP in the *IL1B* gene solely. New studies performing greater genetic coverage strategies of the *IL1B* gene with a larger sample size will be able to ascertain the participation of this SNP in the humoral response against *P. vivax*.

Conclusions

This study suggests that SNPs in the *IL1β* gene may be associated with antimalarial antibodies, particularly against specific variants of *P. vivax* CSP. However, this study evaluated only one SNP (rs16944) in this gene. Further studies with a larger population size and more genetic variants will help to elucidate the mechanisms that control the immune response against *P. vivax*.

Authors' contributions: MPC, CRBD, JOF and RLDM designed the study, performed the research, evaluated the data and collaborated in the manuscript writing. GCC, LMSM, LRPR, TSP, APDR and JEGA collaborated in the sample collection and performed the laboratory tests. MPC, MRP, GCC and ARSB performed the statistical analysis and analyzed the data. MPC, CRBD, LMSM, JOF and RLDM critically reviewed the manuscript. All authors read and approved the final manuscript. MPC is the guarantor of the paper.

Acknowledgements: We acknowledge all the individuals who enrolled in this study. We are grateful to Carlos Cavasini, Luciana Moran and Valeria Fraga for their technical assistance and to Norman Ratcliffe for the English revision of the manuscript.

Funding: This study was supported by grants from the Brazilian Agencies: Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES – DS and PDSE) – Finance Code 001.

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval: This study was approved by the Research Ethics Committee of the Júlio de Mesquita Filho São Paulo State University (UNESP) under protocol number 331 162.

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